Genetic diversity of *Apis cerana cerana* populations in the Yimeng Mountains, Shandong province, East China

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Abstract: 【Aim】 *Apis cerana cerana* is an important germplasm resource and pollinator. This study aims to explore the genetic diversity and population structure of *A. cerana cerana* in the Yimeng Mountains, Shandong province, East China so as to provide a theoretical foundation for the utilization and conservation of germplasm resource for *A. cerana cerana*. 【Methods】 In this study, 114 broods of *A. cerana cerana* from 7 localities in the Yimeng Mountains were selected and 36 morphological indexes were measured according to Ruttner's analytical method. And the genetic diversity of *A. cerana cerana* in the Yimeng Mountains was evaluated with 11 microsatellite loci. 【Results】 The results of morphological analysis showed that the average body length and the average forewing length of *A. cerana cerana* foraging workers in the Yimeng Mountains were 12.064 – 13.351 mm and 8.198 – 8.694 mm, respectively. Fifty-eight alleles were found in the 11 microsatellite loci. The number of alleles per locus ranged from 2 to 17. The average expected heterozygosity and polymorphism information content (*PIC*) of all loci were 0.3115 and 0.2872, respectively. The average number of alleles in all the populations ranged from 2.4545 (ST-AQ) to 4.0000 (BHY), and the average expected heterozygosity ranged from 0.1916 to 0.3397. The average genetic differentiation measured as an *Fst* value was 0.048. The Nei's genetic distance ranged from 0.0092 (XL-DLZ to XZ-XLZ) to 0.1000 (XL-DLZ to XL-DJW). Besides, the cluster analysis results showed that all the populations in the Yimeng Mountains could be grouped into three categories. 【Conclusion】 The population structure of *A. cerana cerana* populations in the Yimeng Mountains is similar. The results suggest that the gene flow occurs in different populations. Compared with the populations in other positions, *A. cerana cerana* in the Yimeng Mountains shows certain specificity in morphology and genetic structure.

Key words: *Apis cerana cerana*; morphology; microsatellite; genetic diversity; Yimeng Mountains

1 INTRODUCTION

The Chinese honeybee, *Apis cerana cerana*, is the geographic subspecies of *Apis cerana*. Compared with the Western honeybee (*Apis mellifera*), it has an acute sense of smell and a strong resistance to mites and can forage a wide range of nectars and pollens (Li *et al.*, 2008). *A. cerana cerana* has a wide distribution in China. However, the qualities of *A. cerana cerana* in China have decreased to less than 10 000 broods because of interspecies competition since the introduction of *A. mellifera*. The Yimeng Mountains in Linyi city, Shandong province, East China, has a temperate continental monsoon climate. This area has sufficient sunshine and the weather is relatively moderate with an annual average temperature of 13.1℃. The Yimeng Mountains has a long history of *A. cerana cerana* rearing with traditional farming methods, such as vat farming.

There are many research methods for examining honeybee genetic diversity. Different methods can reveal genetic information from different angles and levels. A morphological method can describe the genetic variation by measuring morphological and phenotypic characteristics (Ruttner, 1988; Li and

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Wang, 2004). The morphological characteristics mainly include body length, length of femur, cubital index, *etc*. All the morphological characteristics are important for line identification. Before the 1960s, morphological analysis was an important method for variety identification and genetic structure analysis in honeybee. Morphological analysis is a common tool for determining honeybee genetic diversity (Ruttner, 1988).

Due to their high polymorphism, locus specificity, abundance and random distribution over the genome and codominant inheritance, microsatellites are most commonly used to assess population structure and diversity (Weigend and Romanov, 2001). The Honeybee Genome Sequencing Consortium reported that there are more than 2 000 microsatellite markers. The average distance between microsatellite markers is 2.1 cM (The Honeybee Genome Sequencing Consortium, 2006). According to FAO recommendations, determining classic genetic distances using neutral, highly polymorphic microsatellite markers is the method of choice for investigating genetic relationships and breed differentiation (Crooijmans *et al.*, 1996). This methodology also provides information for establishing preservation priorities for livestock breeds (Barker, 1999). Estoup *et al.* (1993) analysed plesiomorphic bee species based on two microsatellite markers. Franck *et al.* (2000) proved that the honeybee in the Near East may be the fourth bloodline of *A. mellifera*.

Analysing the genetic diversity of *A. cerana cerana* in the Yimeng Mountains could provide a theoretical foundation for the research on the conservation and reasonable utilization of honeybee populations. In this study, essential data of 11 microsatellite markers and 36 morphological indexes from seven locations in Yimeng Mountains are provided. The results may be useful to understand the genetic differentiation in this important genetic resource and contribute to more efficient conservation.

2 MATERIALS AND METHODS

2.1 Biological specimens

We chose the localities based on the honeybee breeding method, which was vat breeding or soil breeding, and all the bees were collected from the field. We chose seven localities, and their detailed information was shown in Table 1.

Table 1 Sampling information of *Apis cerana cerana* in the Yimeng Mountains, Shandong

Populations	Sampling locality	Sampling date	Longitude and latitude	Elevation (m)	Number of broods
XZ-XLZ	Xiaoliuzhuang Village, Xuezhuang Town, Linyi City	2017/7/7	35°21′36″N, 118°10′26″E	200	20
DTZ-ZJZ	Zhoujiazhuang Village, Datianzhuang Town, Linyi City	2017/7/7	35°27′38″N, 118°0′45″E	350	15
BHY	Baihuayu, Linyi City	2017/7/7	35°33′31″N, 117°55′35″E	550	20
XL-DJW	Dajiawan Village, Xili Town, Zibo City	2017/7/29	36°0′35″N, 118°14′58″E	338	15
XL-DLZ	Daliuzhuang Village, Xili Town, Zibo City	2017/7/29	36°0′12″N, 118°15′14″E	385	15
ZZ-ZHZ	Zhonghanzhuang Village, Zhongzhuang Town, Zibo City	2017/7/29	36°2′26″N, 118°17′9″E	316	10
ST-AQ	Anqing Village, Sitou Town, Weifang City	2017/7/29	36°18′43″N, 118°31′32″E	174	19

To obtain similar aged bees, we collected samples at the door of the beehive. In total, 114 broods from seven localities in the Yimeng Mountains were collected. Ten foraging workers were collected from each brood, and the samples were placed in 75% ethyl alcohol as materials for the following morphological study. Moreover, one more worker bee was collected from each brood and placed in 95% ethyl alcohol as materials for the following microsatellite study. DNA was extracted according to reports by Ji *et al.* (2009) and then preserved at −20℃ after the content and purity were tested.

2.2 Morphological index measurement and analysis

Ten bees were selected randomly from each brood. Then, all the bees were dissected and their 36 morphological indexes, including body length, length of femur, cubital index, *etc*., were measured. The measurement standards referred to Wang (2007).

2.3 Microsatellite primer selection and multiplex PCR amplification

According to the results of Ji *et al.* (2014), 11 pairs of microsatellite primers were selected (Table 2). The 11 pairs of microsatellite primers were labelled with FAM (blue), HEX (green) or TAMRA (yellow) fluorochromes at the 5′-terminus. Then, the primers were combined according to their base length (Ji *et al.*, 2014). The primer combination information was included in Table 3. All the primers were synthesized by Sangon Biotech (Shanghai). PCR products were obtained in a 20 μL

Table 2 Microsatellite primers used in this study

Locus	Primers	Primer sequences (5′ – 3′)	Repetitive unit	GenBank accession no.
K	29-CL1229. Contig33F	CACAAGGAGTTCTTTCTCGACAT	(AT)8	NW_003377929. 1
	29-CL1229. Contig33R	TTCCACCGTATTGGAATTGATTA		
L	33-CL1360. Contig14F	AGTTCGACAGACCAAGCTGTAAG	(TG)6	NW_003378041. 1
	33-CL1360. Contig14R	CTTGTGCGAACATAGAACAAAGA		
M	1-CL1229. Contig27F	ATTCTACGATACGGCACGTTACA	(AT)8	NW_003378122. 1
	1-CL1229. Contig27R	ATTGTGAGTGGGTACAGTTTCGT		
P	7-CL1278. Contig1F	TGTGATAGAATCGTGTGTCTTACG	(TG)7	NW_003378123. 1
	7-CL1278. Contig1R	GCCAATGTAATAACGATAGTGCC		
Q	11-CL133. Contig41F	AGAGGGGGAAGAAAAGAAAAGAA	(TCG)6	NW_003377943. 1
	11-CL133. Contig41R	GACCGGTTAATCACTGGTGTC		
R	14-CL1549. Contig3F	CGTCCAAGTCTGTACTCCAGC	(CCG)5	NW_003378143. 1
	14-CL1549. Contig3R	AGTAAGTGACGAACACCGTATCG		
S	17-CL1462. Contig5F	AACAAATAGGCCTCGTTAATGTTT	(AC)9	NW_003377909. 1
	17-CL1462. Contig5R	CGGCTGCTTCTTTCTCTTTTTAT		
T	18-CL1470. Contig3F	AATCGATAGACGATGAATTTGGA	(TAA)5	NW_003377909. 1
	18-CL1470. Contig3R	GGACGAAATGGATCAAAGTAAGA		
X	39-CL308. Contig6F	TAGAGGTGGAAGGCAATTATCTG	(AT)6	NW_003377999. 1
	39-CL308. Contig6R	AAATTATGTGATTATTACAATGAGTACC		
W	37-CL1293. Contig1F	GATAGTTACACGCCGCTTAAATG	(AT)8	NW_003377999. 1
	37-CL1293. Contig1R	GATTACTAGATCCAGCGCACATC		
γ	51-CL1114. Contig15F	TCGAATGTTAGGATTTTCGTGAT	(CG)6	NW_003378039. 1
	51-CL1114. Contig15R	ATGCGAGTACGTGTGAATGTATG		

Table 3 Microsatellite primer combinations for multiplex PCR in this study

Locus	Primers	Genes	GenBank accession no.	Modification	Product length (bp)	Annealing temperature (℃)	Chromosome position	Combination used
P	7-CL1278. Contig1	*LOC*410851	NW_003378123. 1	FAM (blue)	159	59. 5	LG2	
γ	51-CL1114. Contig1	*Mblk*-1	NW_003378039. 1	HEX (green)	145	59. 9	LG15	P&γ&X
X	39-CL308. Contig6	*LOC*412784	NW_003377999. 1	TAMRA (yellow)	114	57. 5	LG9	
S	17-CL1462. Contig5	*complexin*	NW_003377909. 1	FAM (blue)	159	59. 9	LG4	
T	18-CL1470. Contig3	*LOC*413936	NW_003377909. 1	HEX (green)	142	59. 6	LG4	S&T&W
W	37-CL1293. Contig1	*LOC*100749790	NW_003377999. 1	TAMRA (yellow)	89	60. 1	LG9	
K	29-CL1229. Contig33	*LOC*100870946	NW_003377929. 1	FAM (blue)	156	60. 1	LG7	K
L	33-CL1360. Contig14	*PMCA*	NW_003378041. 1	FAM (blue)	151	59. 7	LG8	L&M
M	1-CL1229. Contig27	*LOC*100870946	NW_003378122. 1	HEX (green)	137	60. 3	LG1	
R	14-CL1549. Contig3	*RfC*4	NW_003378143. 1	FAM (blue)	150	60. 2	LG3	R&Q
Q	11-CL133. Contig41	*LOC*100577278	NW_003377943. 1	HEX (green)	136	60. 1	LG3	

reaction volume containing 10 × Buffer 2. 0 μL, $MgCl_2$ (25 mmol/L) 1. 0 μL, dNTPs (10 mmol/L) 0. 5 μL, forward primer (10 pmol/μL) 1. 0 μL, reverse primer (10 pmol/μL) 1. 0 μL, Taq DNA polymerase (5 U/μL) 0. 2 μL, worker bee DNA template (1. 0 ng/μL) 1. 0 μL and ultrapure water 13. 3 μL. PCR was performed according to the following program: (1) 5 min at 95℃; (2) 30 cycles of 50 s at 95℃, 50 s at 59 – 62℃, and 50 s at 72℃; and (3) 10 min at 72℃. Six microliters of the products from the last step was used to perform agarose gel electrophoresis for 20 min at 120 V to examine the PCR products. Short tandem repeat (STR) genotyping of the PCR products was performed by Sangon Biotech and the atlas files were generated by GeneMapper 4. 0.

2. 4 Statistical analysis

Discriminant analysis and clustering analysis were performed based on the morphological indexes with SPSS 22. 0. The allele frequency, and observed

heterozygosity (*Ho*) and expected heterozygosity (*He*) (Nei, 1987) for each population across the loci and for each locus across the populations were estimated with the Microsatellite-Toolkit for Excel based on the results of microsatellite genotyping. The polymorphism information content (*PIC*) for each locus was obtained according to Botstein *et al*. (1980). The *F*-statistics indexes (Wright, 1978) were estimated in the form of *Fit*, *Fst* and *Fis*, the sample-based, respective estimators of these parameters proposed by Weir and Cockerham (1984), as implemented in the FSTAT program. The significance of the *F*-statistics was determined from permutation tests with the sequential Bonferroni procedure applied over the loci (Goudet, 2002). The *Fst* values among the populations and effective individual migration in each generation were calculated with the GENEPOP program (Raymond and Rousset, 1995). The Nei's genetic distance and genetic identity were calculated with Popgene 32. The phylogenetic trees were created based on Nei's genetic distances using the neighbour-joining method. The genetic structure of the population was estimated with the Structure program and the optimal cluster numbers were calculated using Structure Harvester (http: // taylor0. biology. ucla. edu/ structureHarvester/). The genetic structure figure was drawn with Distructure.

3 RESULTS

3.1 Morphological characteristics of *A. cerana cerana* in the Yimeng Mountains

The bees were dissected and were made specimens. We measured and counted the data. The results showed that the body length of *A. cerana cerana* of seven populations from the Yimeng Mountains was 12.064 – 13.351 mm, which was higher than the reported value in the literature (11.0 – 13.0 mm) (China National Commission of Animal Genetic Resources, 2011). The average body length of the XL-DLZ population was 13.351 mm. The average forewing length of the seven populations was 8.198 – 8.694 mm and the average forewing width was 2.828 – 2.984 mm. The average cubital index was 3.239 – 3.688. All the morphological information was presented in Table 4.

3.2 Morphological index discriminant analysis of *A. cerana cerana* populations in the Yimeng Mountains

The Wilk's lambda value from the stepwise discriminant analysis was used to classify the 36 morphological indexes from the 114 broods. Then, a discrimination function scatter diagram was drawn (Fig. 1). *A. cerana cerana* populations from different

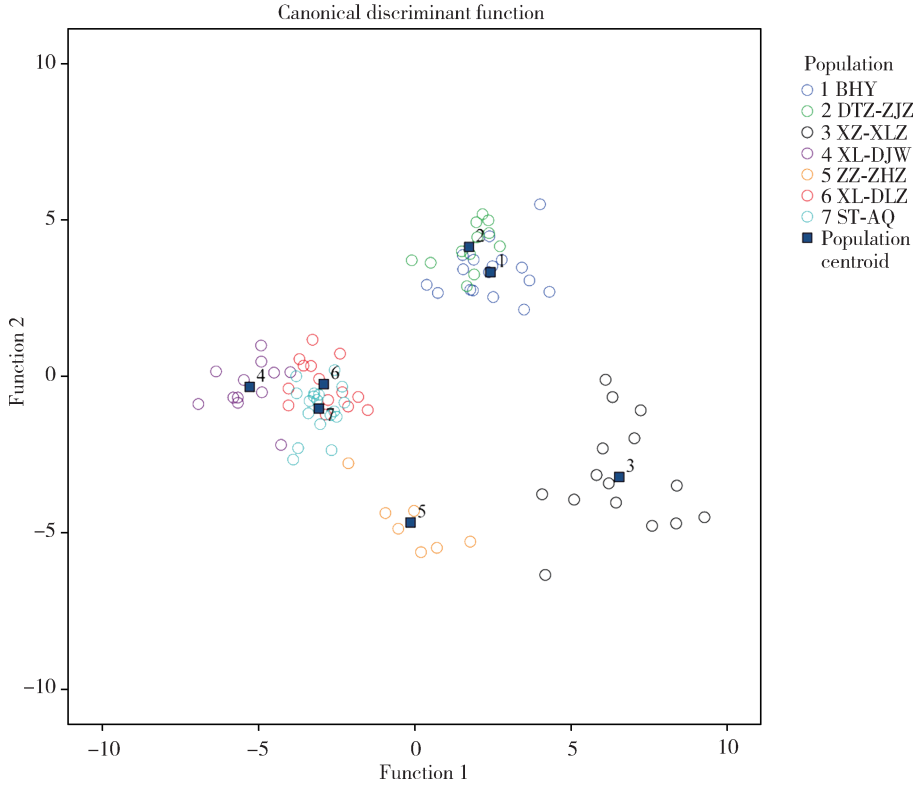

Fig. 1　Discriminant analysis of 36 morphological indexes of *Apis cerana cerana* populations in the Yimeng Mountains

Table 4 Morphometric results of *Apis cerana cerana* populations in the Yimeng Mountains

Characters	BHY	DTZ-ZJZ	XZ-XLZ	XL-DJW	ZZ-ZHZ	XL-DLZ	ST-AQ
Body length (mm)	12.797 ± 0.652	12.191 ± 0.476	12.064 ± 0.393	13.078 ± 0.768	13.050 ± 0.442	13.351 ± 0.533	12.619 ± 0.736
Length of femur (mm)	2.799 ± 0.050	2.813 ± 0.045	2.733 ± 0.106	2.830 ± 0.069	2.781 ± 0.046	2.790 ± 0.057	2.785 ± 0.060
Tibia length (mm)	2.839 ± 0.039	2.866 ± 0.054	2.831 ± 0.091	2.845 ± 0.060	2.752 ± 0.074	2.786 ± 0.059	2.810 ± 0.074
Length of metabasitarsus (mm)	1.797 ± 0.047	1.830 ± 0.062	1.766 ± 0.058	1.857 ± 0.059	1.819 ± 0.059	1.775 ± 0.107	1.786 ± 0.045
Width of metabasitarsus (mm)	1.078 ± 0.024	1.086 ± 0.024	1.064 ± 0.032	1.052 ± 0.031	1.047 ± 0.029	1.015 ± 0.051	1.032 ± 0.047
Forewing length (mm)	8.641 ± 0.149	8.694 ± 0.212	8.602 ± 0.092	8.214 ± 0.235	8.244 ± 0.094	8.384 ± 1.131	8.198 ± 0.154
Forewing width (mm)	2.939 ± 0.047	2.984 ± 0.150	2.879 ± 0.041	2.828 ± 0.065	2.856 ± 0.029	2.837 ± 0.035	2.869 ± 0.057
Length of cubitus (mm)	0.517 ± 0.022	0.508 ± 0.022	0.509 ± 0.019	0.506 ± 0.021	0.502 ± 0.017	0.520 ± 0.022	0.508 ± 0.027
Width of cubitus (mm)	0.142 ± 0.013	0.142 ± 0.007	0.139 ± 0.009	0.143 ± 0.011	0.143 ± 0.015	0.152 ± 0.012	0.158 ± 0.016
Cubital index	3.676 ± 0.418	3.574 ± 0.196	3.688 ± 0.274	3.558 ± 0.257	3.558 ± 0.352	3.442 ± 0.280	3.239 ± 0.323
Length of hair on tergum (mm)	0.015 ± 0.003	0.014 ± 0.003	0.018 ± 0.005	0.010 ± 0.002	0.012 ± 0.002	0.016 ± 0.002	0.012 ± 0.003
Width of tergum 3 (mm)	1.775 ± 0.044	1.717 ± 0.144	1.765 ± 0.073	1.794 ± 0.078	1.785 ± 0.085	1.724 ± 0.039	1.765 ± 0.066
Width of tergum 4 (mm)	1.762 ± 0.147	1.722 ± 0.055	1.738 ± 0.083	1.765 ± 0.085	1.787 ± 0.064	1.710 ± 0.036	1.754 ± 0.065
Width of tomentum on tergum 4 (mm)	0.028 ± 0.005	0.029 ± 0.005	0.030 ± 0.010	0.027 ± 0.009	0.032 ± 0.004	0.0031 ± 0.005	0.035 ± 0.004
Width of dark stripe on tergum 4 (mm)	0.094 ± 0.007	0.091 ± 0.005	0.081 ± 0.008	0.095 ± 0.007	0.087 ± 0.004	0.091 ± 0.006	0.082 ± 0.005
Pigment of scutum 1	5.695 ± 0.555	5.147 ± 0.366	5.453 ± 0.946	5.127 ± 0.822	5.010 ± 1.550	5.240 ± 0.698	5.500 ± 0.432
Pigment of scutum 2	0.705 ± 0.640	0.093 ± 0.149	1.729 ± 0.971	0.020 ± 0.041	1.090 ± 0.285	0.167 ± 0.282	0.221 ± 0.381
Pigment of tergum 2	6.275 ± 0.609	6.060 ± 0.264	5.626 ± 0.681	6.673 ± 0.524	5.470 ± 0.678	6.373 ± 0.422	6.468 ± 0.545
Pigment of tergum 3	5.485 ± 0.499	5.420 ± 0.484	3.974 ± 1.290	5.907 ± 0.459	3.340 ± 1.434	5.6640 ± 0.372	5.647 ± 0.459
Pigment of tergum 4	4.930 ± 0.635	4.992 ± 0.049	3.258 ± 1.353	4.940 ± 0.325	2.540 ± 1.163	5.000 ± 0.160	4.884 ± 0.297
Pigment of labrum 1	3.900 ± 0.620	4.287 ± 0.146	2.906 ± 0.974	1.987 ± 0.757	1.780 ± 0.274	2.580 ± 0.841	1.763 ± 0.860
Pigment of labrum 2	2.020 ± 0.561	1.753 ± 0.358	1.347 ± 0.232	1.513 ± 0.498	1.644 ± 0.407	1.533 ± 0.482	1.500 ± 0.403
Vein angle A4 (°)	33.498 ± 1.302	33.371 ± 0.978	33.159 ± 1.296	32.477 ± 1.529	33.841 ± 1.724	32.895 ± 2.192	33.685 ± 1.163
Vein angle B4 (°)	108.955 ± 1.946	107.236 ± 3.359	109.238 ± 2.216	104.360 ± 5.379	105.251 ± 2.426	106.292 ± 3.349	105.949 ± 3.112
Vein angle D7 (°)	97.220 ± 1.395	96.845 ± 1.776	97.392 ± 1.613	93.247 ± 2.075	93.682 ± 2.209	93.797 ± 2.177	96.107 ± 1.928
Vein angle G18 (°)	83.036 ± 1.739	82.988 ± 2.213	82.194 ± 1.276	82.296 ± 2.385	82.099 ± 1.836	81.016 ± 2.222	85.029 ± 2.706
Vein angle K19 (°)	76.749 ± 2.439	77.984 ± 1.935	76.771 ± 1.655	77.959 ± 2.861	75.498 ± 1.432	75.807 ± 5.557	76.144 ± 2.096
Vein angle O26 (°)	31.532 ± 1.739	32.337 ± 2.599	32.989 ± 2.240	35.841 ± 2.301	34.816 ± 1.997	35.389 ± 2.107	34.871 ± 2.818
Vein angle E9 (°)	22.172 ± 1.017	23.102 ± 0.874	22.859 ± 2.842	20.636 ± 1.935	20.134 ± 1.263	20.758 ± 1.418	21.302 ± 2.470
Vein angle L13 (°)	15.419 ± 1.137	15.001 ± 0.617	14.858 ± 0.585	16.301 ± 2.573	15.749 ± 0.886	15.138 ± 0.815	16.382 ± 0.964
Vein angle J16 (°)	99.197 ± 2.366	100.749 ± 8.611	99.647 ± 1.462	94.465 ± 3.464	95.013 ± 3.255	93.297 ± 4.583	96.405 ± 2.627
Vein angle N23 (°)	80.776 ± 2.801	78.947 ± 4.171	80.314 ± 2.452	80.736 ± 2.233	80.400 ± 1.804	78.827 ± 2.955	80.567 ± 2.451
Vein angle J10 (°)	48.870 ± 2.396	49.550 ± 3.193	48.507 ± 1.967	49.379 ± 3.772	50.730 ± 2.297	49.395 ± 2.948	52.465 ± 3.221
Length of sternum 3 (mm)	2.400 ± 0.052	2.451 ± 0.051	2.361 ± 0.064	2.444 ± 0.043	2.386 ± 0.051	2.398 ± 0.037	2.409 ± 0.051
Length of sternum 6 (mm)	2.301 ± 0.047	2.266 ± 0.039	2.249 ± 0.050	2.271 ± 0.042	2.233 ± 0.082	2.276 ± 0.026	2.240 ± 0.045
Width of sternum 6 (mm)	2.755 ± 0.071	2.750 ± 0.051	2.649 ± 0.062	2.731 ± 0.059	2.697 ± 0.066	2.762 ± 0.069	2.660 ± 0.074

Data in the table are mean ± *SE*.

positions were clustered based on the centroid of the different populations to estimate the morphological distances. As shown in the Fig. 1, the BHY and DTZ-ZJZ populations clustered together, while the XL-DJW, XL-DLZ and ST-AQ populations clustered. However, the XZ-XLZ population was far from the other populations, and the different broods in this population were spread out.

3.3 Intra-population genetic variation of *A. cerana cerana* populations in the Yimeng Mountains

A total of 58 alleles were detected in the seven populations in the Yimeng Mountains with 11 microsatellite markers. The number of alleles, size of the alleles, observed heterozygosity (*Ho*) and expected heterozygosity (*He*) and *PIC* for each locus were listed in Table 5.

Table 5 Genetic variation information in seven *Apis cerana cerana* populations of the Yimeng Mountains based on 11 microsatellite markers

Locus	Number of alleles	Size of alleles (bp)	*He*	*Ho*	*PIC*
P	14	158 – 172	0.8456	0.9115	0.8233
γ	17	127 – 152	0.7533	0.7788	0.7202
X	3	111 – 113	0.2717	0.0000	0.2384
S	2	147 – 148	0.0519	0.0000	0.0504
T	4	132 – 135	0.5784	0.5841	0.4853
W	2	264 – 265	0.0175	0.0000	0.0172
K	2	257 – 258	0.0346	0.0000	0.0339
L	2	151 – 152	0.0175	0.0000	0.0172
M	6	140 – 156	0.4337	0.3009	0.4072
R	3	142 – 148	0.3949	0.4771	0.3395
Q	3	102 – 106	0.0270	0.0273	0.0265
Mean	5.2727		0.3115	0.2800	0.2872

He: Expected heterozygosity; *Ho*: Observe heterozygosity; *PIC*: Polymorphism information content. For locus infromation, see Table 2.

The number of alleles per locus ranged from 2 to 17, and the average number of alleles observed in the 11 microsatellite loci was 5.2727. Across the populations, both the W and L loci had the lowest *Ho*, *He* and *PIC*. In contrast, the P locus had the highest *Ho*, *He*, and *PIC* values (0.8456, 0.9115 and 0.8233, respectively). The average value of *Ho*, *He* and *PIC* were 0.3115, 0.2800 and 0.2872, respectively.

The average number of alleles and *Ho* and *He* for each population across the 11 loci were shown in Table 6. The BHY population had the highest average number of alleles. Across the 11 loci, the XL-DLZ population had the highest *Ho* value (0.3119), and BHY had the highest *He* value (0.3397). It was interesting that the ST-AQ population had the lowest average number of alleles, *Ho* and *He*.

Table 6 Average genetic variation information in seven *Apis cerana cerana* populations in the Yimeng Mountains

Populations	Number of alleles	Average *Ho*	Average *He*
XL-DLZ	2.8182	0.3119	0.2856
XL-DJW	2.9091	0.2611	0.2715
ST-AQ	2.4545	0.1798	0.1916
ZZ-ZHZ	3.0000	0.2536	0.2790
DTZ-ZJZ	2.7273	0.2603	0.2688
XZ-XLZ	2.9091	0.2673	0.2781
BHY	4.0000	0.2823	0.3397

3.4 Inter-population genetic diversity of *A. cerana cerana* populations in the Yimeng Mountains

The results from the *F*-statistics analysis were shown in Table 7. The genetic diversity of seven populations was tested by using the *Fit*, *Fst* and *Fis* values of each locus. The negative *Fis* values for some populations indicated an excess of heterozygous genotypes with respect to the expected value. For the whole population, the inbreeding index (*Fst*) was 0.048 ($P < 0.05$); all of the loci supported this result, except for the W and L loci. The average *Fit* value was 0.082.

Table 7 *F*-statistics analysis of seven *Apis cerana cerana* populations in the Yimeng Mountains

Locus	*Fit* = *F*	*Fst* = θ	*Fis* = *f*
P	−0.079 ***	0.041 *	−0.124 ***
γ	−0.013	0.056 *	−0.074 ***
X	1.000 ***	0.069 *	1.000 ***
S	1.000 ***	−0.027 *	1.000 ***
T	−0.045 ***	0.045 *	−0.094 ***
W	1.000 ***	0.004	1.000 ***
K	1.000 ***	−0.024 *	1.000 ***
L	1.000 ***	0.004	1.000 ***
M	0.294 ***	0.032 *	0.270 ***
R	−0.163 ***	0.071 *	−0.252 ***
Q	−0.006 ***	0.027 *	−0.034 ***
Mean	0.082 ***	0.048 *	0.036 ***
Standard deviation	0.114	0.005	0.119

F: The deviation between the actual frequency and theoretical frequency of genotype in the total population; θ: The relationship between two gametes in each subpopulations; *f*: The deviation between the actual frequency and theoretical frequency in the subpopulation. Mean estimates from jack-knife over loci. Significance of *F*-statistics was done using Bonferroni permutations based on 1 000 resamplings, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. For locus information, see Table 2.

The Nei's genetic distance between all the populations was calculated according to the allelic

numbers and the frequency of the 11 loci (Table 8). The Nei's genetic distance ranged from 0.0092 (XL-DLZ to XZ-XLZ) to 0.1000 (XL-DLZ to XL-DJW). The genetic identity ranged from 0.9521 (BHY to ZZ-ZHZ) to 0.9908 (XZ-XLZ to XL-DLZ).

Table 8 Nei's genetic distance (lower triangle) and the genetic identity (upper triangle) between *Apis cerana cerana* populations in the Yimeng Mountains

Populations	XL-DLZ	XL-DJW	ST-AQ	ZZ-ZHZ	DTZ-ZJZ	XZ-XLZ	BHY
XL-DLZ		0.9901	0.9805	0.9715	0.9724	0.9908	0.9790
XL-DJW	0.1000		0.9725	0.9757	0.9707	0.9898	0.9766
ST-AQ	0.0146	0.0279		0.9636	0.9603	0.9776	0.9532
ZZ-ZHZ	0.0289	0.0246	0.0371		0.9588	0.9691	0.9521
DTZ-ZJZ	0.0280	0.0298	0.0405	0.0421		0.9775	0.9738
XZ-XLZ	0.0092	0.0102	0.0227	0.0314	0.0227		0.9803
BHY	0.0212	0.0237	0.0479	0.0491	0.0266	0.0199	

3.5 Cluster analysis of *A. cerana cerana* populations in the Yimeng Mountains

In this study, a dendrogram was created using the neighbour-joining method to show the phylogenetic relationships among the seven *A. cerana cerana* populations based on Nei's genetic distances (Fig. 3).

The genetic diversity of the seven populations was estimated with the Structure program. The length of the burn-in period was 20 000, and the number of MCMC reps after burn-in was 100 000. The predicted clusters (K value) were 2 – 6, and the number of iterations was 10. The result was submitted to Structure Harvester to obtain the clusters (optimum K value). In this study, the cluster was K = 4 (Fig. 2). The population genetic structure figure was obtained with the Disturct program (Fig. 3). As shown in the figure, the XL-DLZ, ST-AQ and XZ-XLZ populations were classified into the same cluster. The DTZ-ZJZ and BHY populations were also grouped. Additionally, the XL-DJW and ZZ-ZHZ populations were classified into the same cluster.

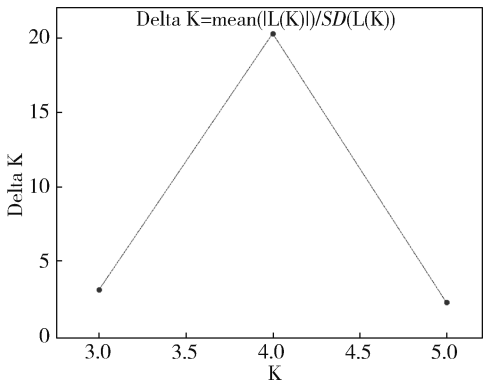

Fig. 2 The predicted clusters of *Apis cerana cerana* populations in the Yimeng Mountains by Structure Harvester

The figure was obtained from online tool Structure Harvester (http://taylor0.biology.ucla.edu/struct_harvest/). K represents the number of population clusters, and the Delta K was the result of software.

4 DISCUSSION

The Yi and Meng Mountains were used as coordinates for the Yimeng Mountains. In this study, the seven sampling localities are mainly located northeast of the Meng Mountain ridge and the Yiyuan country mountain basin. The altitude of these localities ranged from 170 m to 550 m, and these localities covered almost all the geographical features in the Yimeng Mountains.

4.1 Morphological specificity of *A. cerana cerana* in the Yimeng Mountains

In this study, *A. cerana cerana* in the Yimeng Mountains had displayed longer body lengths, shorter tibias, a broader basitarsus and a shorter forewing. All these morphological characteristics were different from documented records (China National Commission of Animal Genetic Resources, 2011). We deduced that the reasons for the morphological specificity were related to the geographical features and ecological environment in the Yimeng Mountains. The Yimeng Mountains belongs to a temperate continental monsoon climate with abundant nectar and pollen plant resources. Studies have shown that a broader basitarsus was beneficial for collecting pollen (Hao, 2014). Additionally, with the altitude and rainfall increasing, the body colour of *A. cerana cerana* was lighter and lighter (Tan *et al.*, 2003; Wang, 2007). The longer body length was related to the high altitude, latitude and annual mean temperature. These results correspond with the results of Ruttner *et al.* (2000).

4.2 Morphological diversity of *A. cerana cerana* in the Yimeng Mountains

The discriminant analysis results showed that

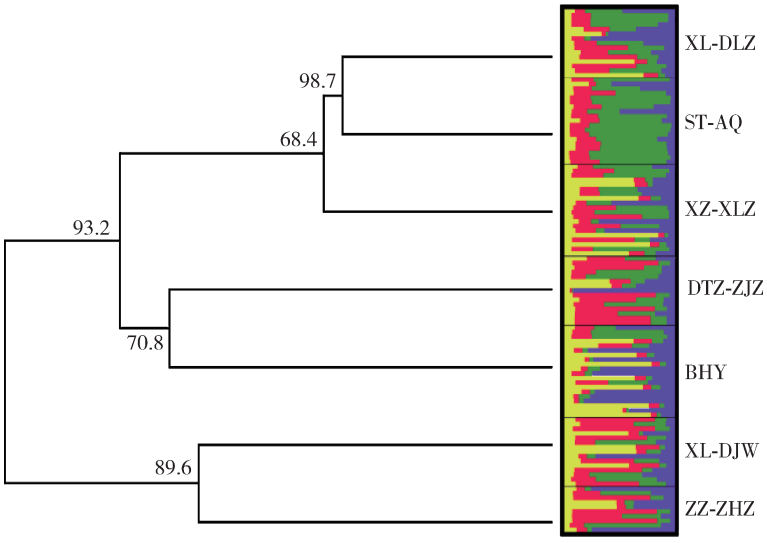

Fig. 3　Dendogram of the phylogenetic relationships and population structure of seven *Apis cerana cerana* populations in the Yimeng Mountains

The phylogentic relationships is based on Nei's genetic distance using neighbor-joining method. The test of phylogeny was carried out using a bootstrap analysis of 1 000 replications; the numbers at the nodes are percentage bootstrap values.

the seven populations could be classified into three classifications. The BHY and DTZ-ZJZ populations clustered together. We thought this was because the two populations are located northeast of the Meng Mountain and the distance between the localities was short. The XZ-XLZ population showed great differences from other populations, and different colonies of its population also had significant differences. The XL-DJW, XL-DLZ, ST-AQ and ZZ-ZHZ populations were all located in the basin of the Yimeng Mountains. Perhaps the similar morphological characteristics were derived from the similar geographical conditions.

4.3　Genetic diversity and relationship analysis with microsatellite markers

We selected 11 microsatellite markers from more than 3 000 microsatellite markers based on Ji *et al.* (2014) transcriptome sequencing results. The 11 microsatellite markers used in the present study are randomly distributed across different chromosomes or linkage groups in the honeybee genome to maintain comparability and representativeness. The alleles with the highest frequency were the most conservative alleles in the populations, and the other alleles were produced by gene mutations during evolution. Therefore, microsatellite polymorphisms could indicate the evolutionary history of the species.

The *PIC* value is a good measure of gene fragment polymorphisms, with the following ranges: (1) $PIC > 0.5$, the locus is a highly polymorphic locus; (2) $0.25 < PIC < 0.5$, the locus is a medium polymorphic locus; and (3) $PIC < 0.25$, the locus is a low polymorphic locus (Vanhala *et al.*, 2001). The *PIC* value is related to the availability and utilization efficiency of a marker; the higher the *PIC* value of a marker, the higher the heterozygote frequency in one population, and the more genetic information it provides. In this study, two loci (P and γ loci) among the 11 microsatellite loci exhibited high polymorphic characteristics, while three loci (T, M and R loci) showed a medium level of polymorphism and six loci (X, S, W, K, L and Q loci) showed a low level of polymorphism (Table 5). The mean *PIC* value across all the loci was 0.2872 (Table 5), which can provide some information for the assessment of genetic diversity.

The effective number of alleles is also a good measure of the genetic variation, especially in conservation genetics studies. The average number of the alleles was 5.2727 across the 11 microsatellite loci in the present study. However, the number of alleles in some populations was small. Still, this result also indicated that the polymorphism information content provided by these 11 microsatellite loci in the seven populations was rich.

Gene heterozygosity, also called gene diversity, is a suitable parameter for investigating genetic variation. Neuman (1993) defined that a polymorphic locus must have a heterozygosity of at least 0.10. However, in our study, five loci among the 11 microsatellite loci exhibited low gene heterozygosity. The result suggests that we may have selected 11 microsatellite markers that were not appropriate for *A. cerana cerana* in the Yimeng Mountains, as they were highly conserved

hereditarily. During the long period of evolution, the bees from the Yimeng Mountains hardly mutated because of the geographic isolation. The mean *He* value can approximately reflect the genetic structure variation; for example, the BHY population had the highest genetic variability (0.3397).

In our study, the average genetic differentiation (*Fst*) value among the broods was 0.048, which indicated that there is no great differentiation among the populations. This result strongly supported the close relationships among the populations. The coefficient *Fis* value indicates the degree of departure from random mating. Positive *Fis* values indicate a significant heterozygote deficit, while negative *Fis* values indicate an excess of heterozygote genotypes with respect to the expected value. In our study, the average *Fis* value was 0.036. However, the values of five loci among the 11 microsatellite markers were 1.000, which also indicated the close relationship among the populations. Additionally, some loci showed a significant deficit of heterozygotes.

The genetic distance could reflect the evolutionary relationship between different populations, and its calculation methods are multifarious (Jiang *et al*, 2007; Yang *et al*., 2007). The Nei's and Reyonld's genetic distance calculation methods are usually used to study population genetic variation using microsatellite DNA polymorphisms. In this study, the Nei's genetic distance between all the populations was calculated. The Nei's genetic distance ranged from 0.0092 (XL-DLZ to XZ-XLZ) to 0.1000 (XL-DLZ to XL-DJW), and the highest value of genetic identity was 0.9908 (XZ-XLZ to XL-DLZ). The results showed that the genetic relationship between XL-DLZ and XL-DJW was close. We speculate that the geographic distance between the two populations is small, leading to high gene flow.

We constructed a phylogenetic tree based on the Nei's genetic distances. The genetic diversity of the seven populations was also estimated with the Structure program. The results showed that all the populations could be grouped into three categories. Interestingly, even though the geographical distance between the XL-DLZ and XL-DJW populations was close, the genetic distance between the two populations was far. We believe that the source of the two populations is different despite of the high gene flow.

The results of genetic diversity were slightly different from the results of morphological index analysis. Although the XL-DLZ and ST-AQ populations were different from the XL-XLZ population in morphological analysis, the XL-DLZ, the ST-AQ and the XZ-XLZ populations were in the same category in microsatellite analysis. In fact, it is generally accepted that the phenotype was determined by genotype. In our study, the clustering results according to genetic distance were not in consistence with that of the analysis of morphology. We thought the reasons were that the samples were not enough or the choice of microsatellite loci was incomplete. So, the RNA-seq and the choice of microsatellite loci would be our next work.

4.4 Conclusion

On the whole, the results from the morphological and microsatellite marker analysis showed that the genetic relationship between *A. cerana cerana* populations from the Yimeng Mountains was close. Compared to the previous research results, our study revealed different morphological characteristics and microsatellite analysis for *A. cerana cerana* from the Yimeng Mountains. The special geographical environment of the Yimeng Mountains and the long history of breeding contributed to the diversity and specificity. Thus, these *A. cerana cerana* populations have unique strain characteristics and should have higher levels of protection and utilization as compared to other populations.

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沂蒙山地区中华蜜蜂种群遗传多样性分析

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摘要: 【目的】中华蜜蜂 *Apis cerana cerana* 是一种重要的种质资源和传粉昆虫,本研究旨在探究山东省沂蒙山地区中华蜜蜂种群遗传多样性和种群结构关系,为沂蒙山地区中华蜜蜂种质资源利用与保护提供理论依据。【方法】本研究通过选取山东省沂蒙山地区 7 个采样地点的 114 群中华蜜蜂采集蜂,根据 Ruttner 的形态学分析方法,对其 36 项形态学指标进行测定;并利用筛选后的 11 对荧光标记微卫星引物,研究其遗传多样性。【结果】形态学分析结果表明,沂蒙山地区中华蜜蜂工蜂的平均体长为 12.064 ~ 13.351 mm,平均前翅长为 8.198 ~ 8.694 mm。11 个微卫星位点共检测到 58 个等位基因,每个位点的等位基因数从 2 ~ 17 不等,7 个群体的平均期望杂合度和多态信息含量分别为 0.3115 和 0.2872,平均等位基因数为 2.4545(ST-AQ) ~ 4.0000(BHY),平均期望杂合度为 0.1916 ~ 0.3397,群体的平均遗传分化系数 *Fst* 为 0.048。Nei 氏遗传距离为 0.0092(XL-DLZ 到 XZ-XLZ)) ~ 0.1000(XL-DLZ 到 XL-DJW)。聚类分析结果表明沂蒙山地区的中蜂可以分成 3 类。【结论】沂蒙山地区各个采样地点之间的中华蜜蜂种群结构接近,表明不同种群之间存在着基因交流,与其他地区中华蜜蜂种群比较,沂蒙山地区中华蜜蜂在形态和遗传结构上存在一定的特异性。

关键词: 中华蜜蜂; 形态学; 微卫星; 遗传多样性; 沂蒙山地区

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